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ACRIDINE DYES AS EFFICIENT REGULATORS OF PHOTOCHEMICAL REACTIONS IN NUCLEIC ACIDS

[Following is the translation of an article by G. B. Zavil'gelskiy, O. N. Rudchenko, and V. V. Danileychenko, Institute of Molecular Biology, AN USSR, Moscow, and the Institute of Epidemiology and Microbiology imeni N. F. Gamaleya, AMN USSR, Moscow, published in the Russian-language periodical Biofizika (Biophysics) 14: 34-42, 1969. It was submitted on 26 Sep 1967.]

In the work it is shown that dyes of the acridine series (atabrine, acridine orange, proflavine, acriflavine, acridine) are effective protectors of infectious DNA from the lethal action of UV-irradiation. The highest protective action is possessed by atabrine, which in DNA inhibits around 96% of lethal photoreactions, and the minimum protective action is possessed by acridine. The acridine dyes protect double-helical and single-strand DNA (DNA of phage T7 and DNA of phage 1φ-7) with equal effectiveness. The value of the protective action of the dyes is proportional to the number of molecules adsorbed on DNA. An investigation is made of the influence of ionic strength, pH, and temperature on the value of the protective action of atabrine. The equilibrium constant is calculated for the process of complex-formation of atabrine with single-strand DNA: $K=7\times10^7$ 1/m. The degree of the protective effect of acridine dyes practically does not depend on the number of lethal photoreactions in DNA which are connected with pyrimidine dimers. It is proposed that acridine dyes decrease the quantum yield of lethal photoreactions of the pyrimidine dimer type and also of reactions with single bases.

In nucleic acids quanta of ultraviolet (UV) light (220-300 nm) induce basically two types of phytochemical reactions: dimerization of neighboring pyrimidine racidals and reactions with single bases of the photohydration and deamination type [1-2]. The quantitative distribution of lethal photoreactions by type is not strictly constant for nucleic acids. A change of conformation of nucleic acid in solution and the use of various UV wavelengths influence the course of photochemical reactions [3-4]. The basic dyes of the acridine series, which form complexes with nucleic acids, also considerably influence the rate of photoreactions with nitrogen bases. This conclusion was made for the first time on the basis of data concerning the protective action of acridine orange during UV-irradiation of infectious DNA [5]. Subsequently it was shown that

acridine orange and proflavine in a complex with two-strand DNA considerably reduce the number of dimers of thymine and inter-thread cross-links which are induced by UV -irradiation [6-7].

In the present work a comparative study is made of the protective action of a number of acridine dyes during UV-irradiation both of double-helical and the single-strand forms of DNA. Conditions are determined for exposing the protective effect and equilibrium constants are calculated for the process of complex-formation of dye with DNA with the manifestation of a protective effect. It is shown that at the maximum binding the dye atabrine inhibits 96% of lethal photoreactions in DNA.

Materials and Methods

In the experiments we used single-chain DNA of enteric phage 1φ-7 and double-chain DNA of T7 phage. Isolation of DNA was carried out from purified concentrates of phages by the phenol method [8]. The resulting preparations of DNA were tested for infectious ability on lysozyme spheroplasts of Escherichia coli, Hfr Kheys, and E. coli C^s (UV-light-sensitive mutant of E. coli C), which was kindly given by R. Sinsheimer (USA). Lysozyme spheroplasts were prepared from a broth culture of E. coli by the method which was described in detail in [9, 10]. For determination of infectious ability the preparation of DNA 1φ-7, diluted in 0.01 M tris-buffer, pH 7.2, was mixed at 37° with an equal volume of spheroplasts, suspended in a concentration of 5×10^8 1/ml in a medium with 0.2% bactopeptone, 0.5 M saccharose, 1.5% serum albumin, and 0.2% $MgSO_4$. After a 10-minute contact the infected spheroplasts were diluted by 10 times with a medium with peptone and 0.7% agar (+10% saccharose and 1.5% albumin) and placed in layers on Petri dishes with a layer of E. coli C washed from the slope.

For determination of the activity of the preparation of DNA of T7 phage 0.2 ml of DNA (usually in a concentration of 20 μg/ml) in 0.01 M phosphate buffer, pH 7.2, was mixed in a suspension of spheroplasts of E. coli C_s, taken in a concentration of $1 \cdot 10^8$ 1/ml in a medium analogous to the previous one, only without magnesium ions, and incubated for 10 min at 37°. Titration of the test was also conducted by the two-layer agar method on a layer of E. coli C. Activity of T7 DNA was not high: around 10^3 active molecules per 4 μg of DNA.

UV-irradiation of DNA preparations was carried out with a BUV-15 tube (wavelength 254 nm). Measurement of dose of UV-irradiation was performed with the help of a UFD-4 dosimeter with a magnesium phototube. Preparations of DNA were irradiated either in watch glasses or in hermetic quartz cuvettes.

In the last case the cuvettes were placed in a water bath at a specific temperature (18-75°). For irradiation the initial preparation of DNA 1φ-7 was diluted with 0.01 M tris, pH 7.2, to a concentration of no more than 0.1 μg/ml. The preparation of DNA T7 was irradiated in an 0.01 M phosphate buffer, pH 7.2, at a concentration no greater than 20 μg/ml.

Acridine dyes (atabrine, proflavine, acriflavine, acridine orange, acridine) from the "Layt" firm were used in the experiments without special purification. Prior to UV-irradiation 0.1--0.5 ml of a specific concentration of dye solution in distilled water was added to the sample of DNA. In order to exclude the photodynamic effect of dyes the stained samples of DNA were preserved in the dark. A special check showed that solutions of dyes in the concentrations used in the work (from 5×10^{-8} to 2×10^{-5} M) and in a layer of 1-2 mm did not exert a noticeable screening action against UV-irradiation from the BUV-15 tube.

Results

Depicted in Fig. 1 are the curves of inactivation of infectious DNA of phages T7 (Fig. 1, A) and 1φ-7 (Fig. 1, B) by UV-light in the presence of various dyes of the acridine series. The position of the dyes in a series based on the degree of protective effect was identical for double- and single-chain forms of DNA molecules. In both cases the most protective effect is possessed by atabrine, an intermediate position (approximately equal) is occupied by proflavine, acriflavine, and acridine orange (AO), and the weakest protective effect is exerted by acridine.

The degree of protective action of dye depends on the number of molecules complexing with DNA. Figure 2 shows the curves of UV-inactivation of DNA of phages T7 (Fig. 2, A) and 1φ-7 (Fig. 2, B) in the presence of various concentrations of atabrine. With a decrease in the concentration of dye the protective effect decreases rapidly. Figure 3 depicts the relationship, depending on concentration of dye, of cross sections of UV-inactivation of DNA of phage 1φ-7 in the absence (σ_K) and in the presence (σ_A) of atabrine. With a concentration of atabrine higher than 1×10^{-5} M a saturation of protective effect is observed - σ_K exceeds σ_A by 25 times. This means that at maximum protection atabrine inhibits the formation of 96% of lethal reactions in DNA. At concentrations of atabrine lower than 5×10^{-8} M the protective effect of dye practically disappears.

With a constant concentration of dye in the solution the number of molecules of dye adsorbed on DNA can be varied by different methods, for example, changing the ionic strength or pH of the solution, increasing the concentration of DNA, changing the temperature during irradiation, etc.

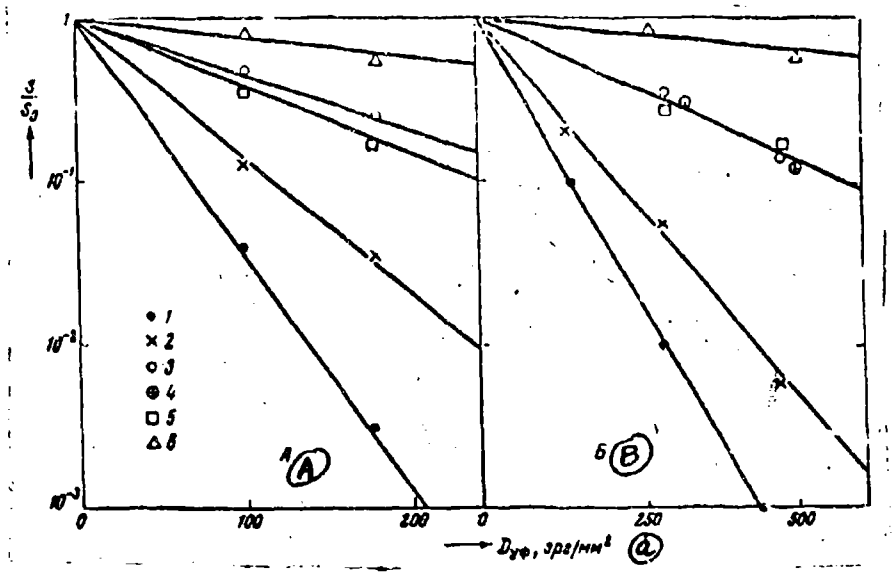

Figure 1. Protective effect of acridine dyes during UV-irradiation (254 nm) of infectious DNA of phage T7 (A) and phage 1φ-7 (B). 1 - control (without dye); 2 - acridine; 3 - proflavine; 4 - acridine orange; 5 - acriflavine; 6 - atabrine. Concentration of dyes: 2×10^{-5} M (A) and 5×10^{-6} (B). DNA of T7 irradiated in a phosphate buffer, pH 7.2 and $\mu = 0.01$ (concentration of DNA 20 μg/ml). DNA of 1φ-7 irradiated in tris pH 7.2, $\mu = 0.01$ (concentration of DNA 0.1 μg/ml). In the presence of acridine (curve 2) irradiation was conducted at pH values of 7.2 and 3.7, since the pH for acridine is equal to 5.6 [21]. In the figure 1, 2, 5, and 6 along the axis of ordinates - the share of molecules of infectious DNA surviving after irradiation (S/S_0) in a logarithmic scale, and along the axis of abscissae - the dose of UV-rays in erg/mm^2. All the experimental points on the chart are the result of averaging of 3- and more-fold repetition.

Key: (a) D_{UV}, erg/mm^2.

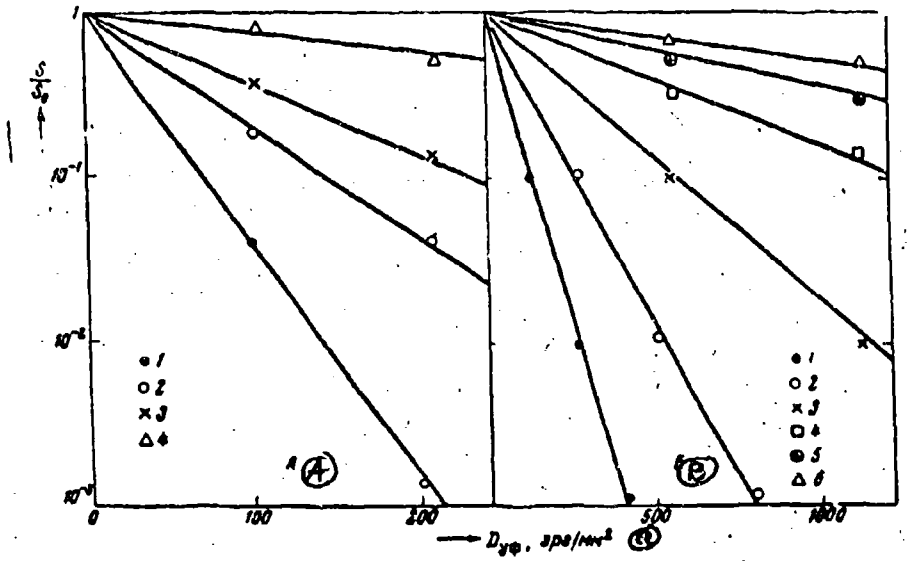

Figure 2. Dependence of value of protective effect on the concentration of atabrine (C_A) during UV-irradiation of infectious DNA of phage T7 (A) and 1φ-7 (B).

A: 1 - control without atabrine; 2 - $C_A = 7 \times 10^{-6}$M; 3 - $C_A = 1 \times 10^{-5}$M; 4 - $C_A = 2 \times 10^{-5}$M. B: 1 - control (without dye); 2 - $C_A = 2 \times 10^{-7}$M; 3 - $C_A = 5 \times 10^{-7}$M; 4 - $C_A = 2 \times 10^{-6}$M; 5 - $C_A = 5 \times 10^{-6}$M; 6 - $C_A = 2 \cdot 10^{-5}$M and $1 \cdot 10^{-5}$M.

[Note: Nos. 4 and 5 - superscripts illegible.]

Key: (a) D_{UV}, erg/mm^2.

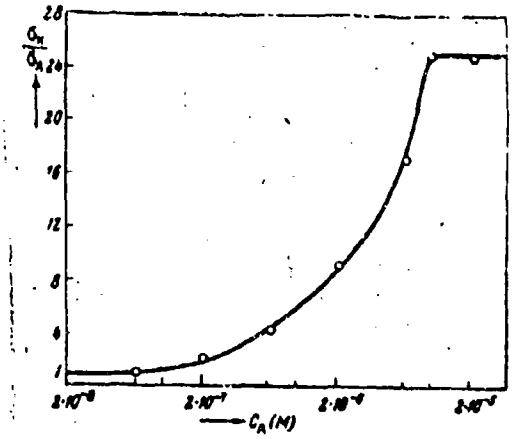

Fig. 3

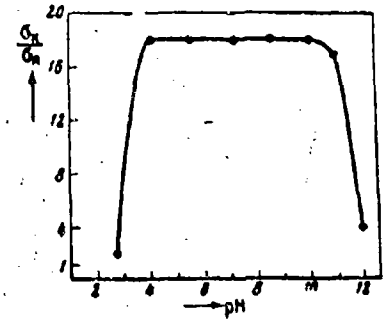

Fig. 4

Fig. 3. Dependence of the ratio of cross sections of UV-inactivation of infectious DNA of 1φ-7 in the absence and in the presence of atabrine (σ_k/σ_A) on the concentration of dye based on the data of Fig. 2, B.

Fig. 4. Dependence of σ_k/σ_A on the pH value. DNA of phage 1φ-7 in a concentration of 0.1 μg/ml in tris (μ=0.01); concentration of atabrine 5×10^{-6}M. The solution of tris was diluted to the required pH value with 1 n HCl or NaOH.

In Fig. 4 is depicted the dependence of protective effect of atabrine (σ_k/σ_A) on the pH value. In the pH interval from 4.0 to 11.0 the protective effect of atabrine is practically not changed. With a pH lower than 3.0 and higher than 11.0 the effectiveness of the dye was reduced strongly. We obtained a similar result with another acridine dye - acriflavine. The weakening of protective action of dyes at extreme pH's is connected with disruption of the process of complexing of dyes of the acridine series with DNA. At a pH lower than 3.0 complexing is disrupted due to intensive protonation of nitrogen bases of DNA (cytosine, adenine). At a pH higher than 11.0 a large share of molecules of dye transform into a neutral form and also lose the capacity to be adsorbed on phosphate groups of DNA. The negative charge of the phosphate groups of DNA can also be shielded by means of increasing the concentration of metal ions in the solution, Na^+ in particular, which also lowers the intensity of complexing of dye with DNA. Simultaneously there is a lowering of protective effect of acridines: at an ionic strength of the solution higher than 0.2 M atabrine practically does not protect.

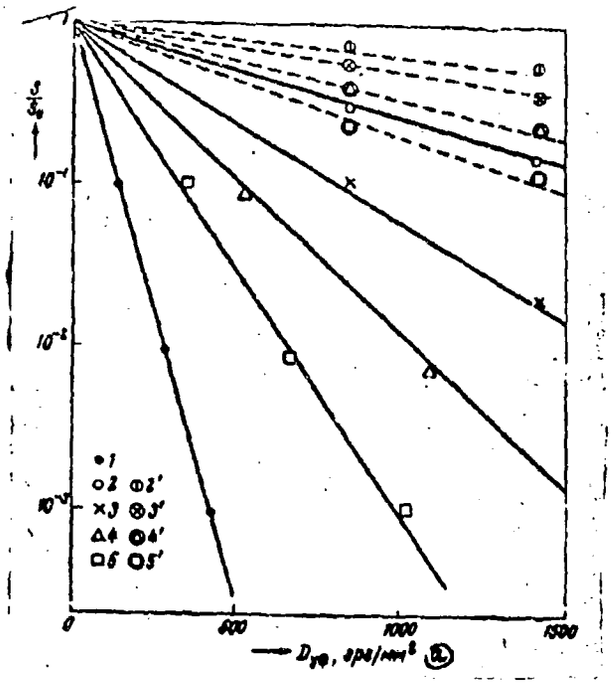

Fig. 5. Influence of temperature during UV-irradiation on the protective effect of atabrine.

1 - control (without atabrine); 2 and 2' - temperature 20°; 3 and 3' - 40°; 4 and 4' - 55°; 5 and 5' - 65°. For all curves $C_A = 3 \times 10^{-6}$M and 2×10^{-5}M correspondingly. Concentration of DNA of 1φ-7 equals 0.1 μg/ml. Irradiation carried out in 0.01 tris with a pH of 7.2.

Key: (a) D_{UV}, erg/mm^2.

With an increase of temperature at the moment of irradiation the share of dye bound with DNA is reduced and correspondingly there is a drop in the protective action of atabrine (Fig. 5). If the initial concentration of dye is selected in the linear area of dependence of σ_k/σ_A on concentration (Fig. 3), then the protective effect begins to weaken already with a comparatively small increase of temperature. For example, at $C_A = 3 \times 10^{-6}$ with a temperature of 40° the protective effect of the dye is weakened by approximately two times in comparison with that at room temperature. If, however, the initial concentration of dye is selected in the area of saturation of effect ($C_A = 2 \times 10^{-5}$M), then the temperature has a weak influence on the grade of curves of UV-inactivation (Fig. 5).

In this case the presence of an excess of molecules of dye in the solution shifts the equilibrium to the side of formation of a complex with DNA even at increased temperatures, which also ensures a high degree of protective effect.

All the above-cited examples of the influence of reactions on the degree of protection of atabrine indicate the necessity of complexing of acridine dyes with DNA for an effective protective action during UV-irradiation, while the extent of protective effect correlates with the degree of filling of DNA with molecules of dye. For each acridine dye there is a characteristic value of maximum filling of DNA (r_m) [11]. The greatest value for r_m is observed for atabrine. Somewhat lesser values of r_m are characteristic for proflavine, acriflavine, and AO, and the minimum value - for acridine. Therefore with an equal concentration for this series of dyes it follows to expect the corresponding distribution of them also based on degree of protective action, which was observed in the experiment (Fig. 1). In addition it is necessary to note that atabrine, in contrast to the other dyes used by us, is photodynamically inactive, which additionally increases its capacity for the protection of DNA from lethal UV-irradiation [12].

An analysis of curves of UV-inactivation of infectious DNA in the presence of dyes makes it possible to calculate the equilibrium constants for bonding of molecules of dye with DNA.

For the reversible reaction of complexing of dye (A) with DNA

$$A + DNA \rightleftarrows [A\text{-}DNA]$$

the constant of equilibrium K is determined under the conditions of homogeneity of the process of binding

$$K = \frac{[A\text{-}DNA]}{[A] \times [DNA]}, \qquad (1)$$

where [A-DNA] - concentration of sites on DNA occupied by dye, DNA - concentration of free sites on DNA, [A] - concentration of free dye [13]. Taking the logarithm of formula (1) we obtain:

$$\lg K = \lg \frac{[A\text{-}DNA]}{[DNA]} - \lg A. \qquad (2)$$

At small concentrations of DNA (less than 0.1 μg/ml) it is possible, with sufficient foundation, to take the value (A) as equal to the initial concentration of dye in the solution.

For the determination of member $\frac{[A\text{-}DNA]}{[DNA]}$ we use data based on the kinetics of UV-inactivation of DNA in the presence of dye. Let N_0 - the complete number of sites on DNA, the damage to any of which is lethal for the macromolecule with the probability W_K. In this case the cross section of inactivation of DNA in the absence of dye may be written in the form:

$$\sigma_K = N_0 \times W_K,$$

and the kinetics of UV-inactivation of DNA will be determined by the formula:

$S/S_0 = e^{-\sigma_K D}$ (D - dose of UV in quanta/mm^2).

Value of equilibrium constant (K) for bonding of atabrine with DNA of 1φ-7 phage

(2) Концентрация атебрина	σ_K/σ_A	K (1/M) (1)
5×10^{-6}	16,0	$8,0\times10^{6}$
2×10^{-6}	9,2	$6,4\times10^{6}$
5×10^{-7}	4,1	$7,5\times10^{6}$

Correspondingly the average value of equilibrium constant is equal to $K = 7.3\times10^6$ 1/m.

Key: (a) Concentration of atabrine; (b) K (1/M).

During complexing of a molecule of dye with a given sector of DNA the probability of damage to this sector is decreased (protective effect) and becomes equal to W_A, and if it is assumed that W_A is constant for all N_0 sites on DNA, then the cross section of UV-inactivation of DNA under the condition of complete protection (all the sites on DNA occupied by molecules of dye) equals:

$$\sigma_A = N_0 \times W_A,$$

and the kinetics of UV-inactivation of DNA will be determined by the formula

$$S/S_0 = e^{-\sigma_A D}$$

Then with a partial filling of sites on DNA by dye the cross section of inactivation will equal:

$$\sigma = N_A \cdot W_A + (N_0 - N_A) \cdot W_K, \qquad (3)$$

where N_A - number of sites occupied by dye, and $(N_0 - N_A)$ - number of free sites on a molecule of DNA.

Having multiplied and divided formula (3) by N_0, we obtain

$$\sigma = \sigma_A \times \frac{N_A}{N_0} + \sigma_K \left(1 - \frac{N_A}{N_0}\right).$$

From here we express the ratio of number of occupied and correspondingly protected sites on DNA to the number of free sites:

$$\frac{[A\text{-}DNA]}{[DNA]} = \frac{N_A}{N_0 - N_A} = \frac{\sigma_K - \sigma}{\sigma - \sigma_A} = \frac{(\sigma_K/\sigma - 1)}{\left(1 - \frac{\sigma_A}{\sigma_K} \times \frac{\sigma_K}{\sigma}\right)}. \qquad (4)$$

Substituting (4) into (2) we obtain:

$$\lg K = \lg \frac{(\sigma_K/\sigma - 1)}{\left(1 - \frac{\sigma_A}{\sigma_K} \times \frac{\sigma_K}{\sigma}\right)} - \lg [A] \qquad (5)$$

Using formula (5) it is possible to calculate the equilibrium constant for the bonding of atabrine with DNA, based on data from Fig. 2, B for DNA of 1φ-7 phage, where the value of maximum protection is obtained. The value of σ_A / σ_K in this case equals 1/25 (Fig. 3). In the table results are presented for a similar calculation for various concentrations of atabrine.

It is necessary to note that the value of K obtained in such a manner is characteristic, not for any complex of dye with DNA, but only for complexes which possess a protective effect.

Discussion of Results

At the present time two possible mechanisms of protective action of acridines are being discussed: 1) the migration mechanism, as a result of which the energy of UV-quanta, adsorbed by the nitrogen base, is transferred to the dye which is found in a complex with it

and is scintillated in the form of a quantum of fluorescence or converted into heat, and 2) the intercalation mechanism, as a result of which the molecules of dye, intercalated between nitrogen bases of DNA, spatially hamper the reaction of photodimerization of neighboring bases [5-7, 12, 14-16]. In the case of the migration mechanism it is possible to expect a lowering of quantum yield of both types of photoreactions in DNA: dimerization of pyrimidines and reactions with single bases. In favor of the intercalation mechanism is the specificity of action of the dye: only the process of photodimerization of pyrimidines is prohibited. In favor of the migration hypothesis data are cited concerning the experimental detection of migration - DNA - acridine dye [17-18].

As can be seen from Fig. 3, atabrine removes up to 96% of lethal photoreactions in single-chain DNA. Under the condition that the intercalation mechanism of protection was correct it would be necessary to assume that 96% of lethal photoreactions in single-strand DNA was made up of dimers of pyrimidines. However, this assumption is found in contradiction with data on the kinetics of induction in single-strand DNA of pyrimidine dimers. Thus, based on the data of David, dimers of thymine determine only 33% of lethal photoreactions in DNA of FKh-174 phage [19]. Indirect data, presented in work [4], connect 50% of lethal photoreactions with dimers of pyrimidines in single-strand DNA of 1φ-7 phage.

Beukers detected an interesting fact: if preliminarily strongly UV-irradiated DNA continues to be irradiated in the presence of proflavine, then the amount of dimers of thymine in DNA is reduced. It is proposed that proflavine, by inhibiting the direct reaction of photodimerization (K_1)

$$T + T \underset{K_2}{\overset{K_1}{\rightleftharpoons}} \hat{TT},$$

does not influence the course of the reversible reaction (K_2) [6]. The presence of such a shift of equilibrium to the side of formation of monomers in the presence of acridines was used by R. and G. Setlow as important proof of the correctness of the intercalation hypothesis [14-16]. However, this effect does not contradict the migration hypothesis also. The fact is that migration from the nitrogen base to the dye is possible only under the condition of overlapping of the triplet state of the base and the singlet or triplet state of the dye. For thymine the band of absorption has a maximum at 265 nm, and a band of phosphorescence - around 450 nm, and overlaps strongly with the band of absorption of acridines (for example, the maximum of absorption of atabrine is around 450 nm) [20]. In a dimer of thymine, in connection with the absence of a band of absorption at 260 nm, the condition of overlapping states is disrupted and correspondingly the migration of energy to the dye is prohibited. The presence of migration from pyrimidine to the dye and the absence of

migration from the dimer to the dye should lead to effective inhibition of a direct reaction without disruption of the reversible reaction and a shift of equilibrium to the side of monomers of pyrimidines.

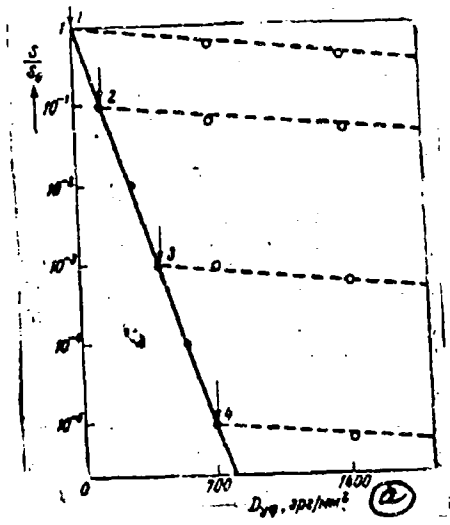

Fig. 6. Protective action of atabrine following dyeing of infectious DNA 1φ-7, irradiated preliminarily up to various degrees of inactivation.

Solid line - inactivation of 1φ-7 DNA in the absence of atabrine; broken line - inactivation of DNA in the presence of atabrine. Concentration of DNA 0.1 μg/ml. Concentration of atabrine 1×10^{-5}M. Tris-buffer, pH 7.2, $\mu = 0.01$. The arrow indicates the moment of addition of atabrine: 1 - from the moment of irradiation; 2 - after a dose of UV of 140 erg/mm^2; 3 - after a dose of UV of 420 erg/mm^2; 4 - after a dose of UV of 700 erg/mm^2.

Key: (a) D_{UV}, erg/mm^2.

The phenomenon of a shift of equilibrium to the side of monomers of pyrimidines can be used for studying the mechanisms of lethal action of UV on DNA [15]. In this case infectious DNA must be irradiated up to a specific level of inactivation, dye added, and irradiation continued. Since the reaction of photodimerization is inhibited here, and the reversible reaction of monomerization proceeds

normally, then it is possible to expect an increase in the number of active infectious molecules of DNA due to the transition of lethal dimers again into monomers. This experiment, conducted on infectious DNA of FKh-174 with proflavine, yielded a negative result; restoration of activity of DNA was not observed /6/. We repeated this experiment on DNA of 10^{-7} with atabrine, assuming that the increased protective capacity of atabrine in comparison with proflavine increases the probability of passage in DNA of the reversible reaction of monomerization. However, as this can be seen from Fig. 6, a negative result was also obtained: restoration of activity in DNA was not observed, residual active DNA was inactivated by UV-light at the same rate as DNA which was irradiated in the presence of atabrine from the very beginning. Consequently, the proposal concerning the specificity of the protective action of atabrine following UV-irradiation of DNA (complete inhibition of the reaction of photodimerization and invariability in the yield of reactions with separate bases) is incorrect. It remains to be proposed that atabrine, and apparently other acridine dyes, lower the quantum yield of lethal photoreactions of any nature both in double-helix and in single-strand forms of DNA molecules.

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